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Enantiomeric Specificity of (-)-2,2',3,3',6,6'-Hexachlorobiphenyl ((-)-PCB 136) Towards Ryanodine Receptor Types 1 and 2

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Abstract

Polychlorinated biphenyls (PCBs) with unsymmetrical chlorine substitutions and multiple *ortho*-substitutions that restrict rotation around the biphenyl bond may exist in two stable enantiomeric forms. Stereospecific binding and functional modification of specific biological signaling targets have not been previously described for PCB atropisomers. We report that (-)-2,2',3,3',6,6'-hexachlorobiphenyl ((-)-PCB 136) enhances the binding of [³H]ryanodine to high affinity sites on ryanodine receptors type 1 (RyR1) and type 2 (RyR2) (EC₅₀s ~ 0.95 μM), whereas (+)-PCB 136 is inactive at 10 μM. (-)-PCB 136 induces a rapid release of Ca²⁺ from microsomal vesicles by selective sensitization of RyRs, an effect not antagonized by (+)-PCB 136. (-)-PCB 136 (500 nM) enhances the activity of reconstituted RyR1 channels 3-fold by stabilizing the open and destabilizing the closed conformational states. The enantiomeric specificity is also demonstrated in intact HEK 293 cells expressing RyR1 where exposure to (-)-PCB 136 (100 nM; 12 hr) sensitizes responses to caffeine, whereas (+)-PCB 136 does not. These data show enantiomeric specificity of (-)-PCB 136 toward a broadly expressed family of microsomal Ca²⁺ channels that may extend to other chiral non-coplanar PCBs and related structures. Evidence for enantioselective enrichment of PCBs in biological tissues that express RyR1 and RyR2 channels may provide new mechanistic leads about their toxicological impacts on human health.

Keywords

polychlorinated biphenyls (PCB); atropisomers; chiral PCB; enantiospecificity; ryanodine receptor type 1; ryanodine receptor type 2; receptor stereospecificity; calcium signaling

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Introduction

Nineteen of the possible 209 polychlorinated biphenyl (PCB) structures exist as pairs of atropisomers (also referred to as enantiomers) that are sufficiently stable to permit separation by gas or liquid chromatography using chiral column matrices (1-3). Stable enantiomeric PCB structures have unsymmetrical chlorine substitution in their respective phenyl rings and possess 3 chlorine atoms in the *ortho*-positions that restrict the degree of rotation about the biphenyl bond. Evidence of enantioselective enrichment of PCB atropisomers in environmental samples (4-10), food (3, 11, 12), as well as biological tissues from animals (13-17) and humans (18, 19) has been widely reported. Although PCB enantiomeric pairs have identical physicochemical properties (*e.g.*, octanol-water partition coefficient, vapor pressure, Hammett's σ constant, etc.), in theory each enantiomer is capable of differential interactions with biological targets (*i.e.*, receptors) which possess three or more functional groups necessary for coordinating stereoselective binding of chiral ligands (20). One hypothesis proposed to account for enantioselective enrichment of PCBs is based on differential rates of biotransformation and metabolism (21-23). In support of this hypothesis, evidence of enantioselective deposition of 2,2',3,3',6,6'-pentachlorobiphenyl (PCB 136) in an experimental mouse model has been published (14-16). Recently, Lehmler and co-workers have shown that (+)-PCB 136 preferentially binds to cytochrome P450 (CYP) enzymes, including isoforms CYP2B and CYP3A present in a microsomal preparation from mouse liver (24, 25).

Enantiospecific binding and functional modification of a biological signaling target has not been previously demonstrated for PCBs. In the present study, we show that (-)-PCB 136, but not (+)-PCB 136, potently sensitizes the activation of two broadly expressed microsomal Ca^{2+} release channels, ryanodine receptors type 1 (RyR1) and type 2 (RyR2). The key findings are: (1) (-)-PCB 136 directly sensitizes activation of RyR1 and RyR2, whereas (+)-PCB 136 does not; (2) (-)-PCB 136 rapidly mobilizes microsomal Ca^{2+} stores by activating RyR1 in the presence of low (resting) levels of cytoplasmic Ca^{2+} , (3) (+)-PCB 136 does not competitively inhibit the actions of (-)-PCB 136; (4) the mechanism by which (-)-PCB 136 promotes RyR activity is to coordinately stabilize the open, while destabilizing the closed, states of the channel; (5) (-)-PCB 136, but not (+)-PCB 136, sensitizes caffeine-induced Ca^{2+} release from intact a human embryonic kidney cell line (HEK 293) expressing RyR1. Enantioselective enrichment of PCBs in biological tissues that express RyR1 and RyR2 channels are likely to provide new mechanistic insights into their toxicologic impacts on human health.

Experimental Procedures

Materials

A racemic mixture of ($\pm$)-PCB 136 was synthesized as previously reported using the Ullmann coupling reaction of 2,3,6-trichloro-1-iodobenzene (26). The racemate was purified using charcoal to remove trace amounts of halogenated dibenzofurans. PCB 136 atropisomers were separated by reversed phase-high pressure liquid chromatography on two serially connected 4.6 $\times$ 250 mm Nucleodex β -PM columns (Macherey-Nagel, Düren, Germany), as described by Haglund (1). The enantiomeric fraction of the PCB 136 atropisomers, a measure of enantiomeric purity, was determined using a HP6890 gas chromatograph equipped with a ^{63}Ni μ -ECD detector and a Chirasil-Dex column (β -cyclodextrin chemically bonded with dimethylpolysiloxane, 25 m $\times$ 0.25 mm ID $\times$ 0.25 μm film thickness, Varian, Palo Alto, CA) (24). Each PCB 136 atropisomer sample was greater than 99% pure. The enantiomeric fractions (EF) of (-)-PCB 136 and (+)-PCB 136 were 0.01 and 1.00, respectively (EF = peak area (+)-PCB 136/[peak area (+)-PCB 136 + peak area (-)-PCB 136]) (27). The identity of RyR-active and RyR-inactive solutions was verified on at

least three separate occasions with the investigators blinded to their respective activity. Ultra pure lipids were used for forming bilayer lipid membranes and were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). [^3H]Ryanodine (50-60Ci/mmol) was purchased from Perkin Elmer (Waltham, MA).

Preparation of RyR enriched microsomes

Microsomal membrane vesicles (junctional sarcoplasmic reticulum) were prepared as previously described from New Zealand White rabbit (28) or C57BL/6J mouse (29) skeletal muscle (RyR1) and Sprague-Dawley rat cardiac muscle (RyR2) (30, 31). The preparations were stored in 10% sucrose, 10mM HEPES, pH 7.4, at -80°C until needed.

[^3H]Ryanodine binding analysis

[^3H]Ryanodine ([^3H]Ry) binds with high affinity and specificity to the open state of RyR1 and RyR2 and therefore provides a convenient measure of ligands that influence channel conformation (31-33). Microsomal membrane vesicles enriched in RyR1 (50 μg protein/ml) were incubated in the presence or absence of the PCB in a solution containing 20mM HEPES, pH7.1, 140mM KCl, 15mM NaCl, 1 μM free Ca^{2+} and 1nM [^3H]Ry. [^3H]Ry binding to RyR2 was measured under the same conditions described for RyR1 except that free Ca^{2+} was adjusted to 200nM in the assay buffer with EGTA (34) to account for the higher sensitivity of RyR2 to Ca^{2+} activation (33). The dependence of PCB activity was tested in separate experiments with incubations of 3h at 37°C or 16h at 26°C . The binding reactions were quenched by filtration through GF/B glass fiber filters and washed twice with ice-cold harvest buffer (20mM Tris-HCl, 140mM KCl, 15mM NaCl, 50 μM CaCl_2 , pH 7.1). Nonspecific binding was determined by incubating microsomes with 1000-fold excess unlabelled ryanodine in the absence or presence of the PCB. Dose-responses of (+)-PCB 136 and (-)-PCB 136 were titrated by adding 5ml of solution from series dilution of 100x stocks in dimethylsulfoxide (DMSO). Controls were measured with the same volume DMSO lacking the PCB. Binding curves were fitted using a non-linear least squares regression model with Origin 7.0 software (OriginLab Corp, Northampton, MA) to calculate EC_{50} .

Microsomal Ca^{2+} flux measurements

Net Ca^{2+} flux across the microsomal membrane vesicles was monitored by the metallochromic dye antipyrilazo-III (APIII) in a buffer consisting of 250 μM in 18.5mM MOPS, 92.5mM KCl, 7.5mM Na-pyrophosphate, pH 7.0 (31). Microsomal vesicles from rabbit skeletal muscle (50 μg /ml protein) were loaded to near capacity by serial additions of 20 μM CaCl_2 in the presence of 1mM MgATP, 5 mM phosphocreatine, and 10 μg /ml creatine phosphokinase. Once the APIII signal returned to baseline ($\sim 100\text{nM}$ extravesicular Ca^{2+}), (+)-PCB 136 or (-)-PCB 136 was introduced into the cuvette at a final concentration of 0.2 to 5 μM to assess their ability to induce Ca^{2+} release. Pharmacological blockade of RyR channels was achieved by the addition of 1 μM ruthenium red prior to introducing PCB. Thapsigargin (200nM) was added at the end of the experiment to block SR/ER Ca^{2+} ATPases (SERCA pumps) and signals were calibrated at the end of each experiment by additions of 20 μM Ca^{2+} from a National Bureau of Standards stock. Experiments were performed in triplicate using three different membrane preparations.

Reconstitution of RyR1 single channels in planar lipid bilayer

RyR1 channels prepared from rabbit skeletal muscle were reconstituted in bilayer lipid membranes (BLM) formed by a mixture of phosphatidylethanolamine:phosphatidylserine:phosphatidylcholine (5:3:2 w/w) across a 200 μm hole drilled in a polysulfone cup (Warner Instruments, Hamden, CT). The BLM partitioned two chambers (*cis* and *trans*) with buffer solution (in mM) 500 CsCl, 1 μM free

Ca²⁺, 2 Na₂ATP and 20 Hepes-Tris (pH 7.4) on *cis*, 50 CsCl, 100 μM free Ca²⁺ and 20 Hepes-Tris (pH 7.4) on *trans*. The addition of microsomal membranes was made to the *cis* chamber that served as the virtual ground, whereas the *trans* chamber was connected to the head stage input of an amplifier (Bilayer Clamp BC 525C, Warner Instruments). Immediately after incorporation of a single RyR1 channel into BLM, the *cis* chamber was perfused with *cis* solution to prevent incorporation of additional channels into the BLM. Single channel gating was recorded at a holding potential of -40mV (applied to the *trans* side) for 1 min before and after addition of PCB 136 atropisomers to the *cis* solution. The amplified current signals, filtered at 1 kHz (Low-Pass Bessel Filter 8 Pole), were digitized and acquired at a sampling rate of 10 kHz (Digidata 1320A, Axon-Molecular Devices, Union City, CA). The channel open probability (P_o), mean open-, and mean closed-dwell times (τ_o and τ_c) were obtained by using Clampfit, pClamp software 9.0 (Axon-Molecular Devices, Union City, CA).

Stable expression of human wild type RyR1 in HEK 293 cells

Human Embryonic kidney (HEK 293) cells were maintained in DMEM medium supplemented with 2mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 1mM sodium pyruvate, 10 % fetal bovine serum at 37°C under 5% CO₂. Cells were transfected with the full length cDNA sequence of RyR1 cloned into pCI-neo expression vector (Promega, Madison, WI) using Lipofectmanin™2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Stably transfected cells were obtained by Geneticin sulphate (G418) selection for two weeks at a final concentration of 500 μg/ml. Single colonies resistant to G418 were then transferred into a 24 well plate and tested for the RyR1 expression using immunofluorescence staining with 34C antibody (Sigma, St. Louis, MO).

Ca²⁺ imaging

HEK 293 cells and *w_t*RyR1-HEK 293 cells were loaded with 5 μM Fluo-4 AM (Invitrogen, CA) at 37°C for 20 min to measure Ca²⁺ transients in an imaging buffer (140 mM NaCl, 5mM KCl, 2mM MgCl₂, 2mM CaCl₂, 10 mM HEPES and 10 mM glucose, pH 7.4 supplemented with 0.05% bovine serum albumin). The cells were washed three times with imaging buffer and additionally incubated for 20 min at room temperature. Dye loaded cells were washed three times with imaging buffer and imaged with an intensified CCD camera (Photon Technologies) with a ×40 objective attached to a Nikon Diaphot Microscope (Nikon, Tokyo, Japan). Fluo-4 was excited at 494 nm and the fluorescence emission was captured at 510 nm. Caffeine was dissolved in the imaging buffer and was focally applied for 5 s using an AutoMate Scientific perfusion system (Berkeley, CA). HEK-293 and *w_t*RyR1-HEK293 cells were incubated with either 100 nM of (-)-PCB 136 or (+)-PCB 136 for 12 h. The bulk perfusion buffer used for imaging the PCB pre-treated cells contained 100 nM (-)- or (+)-PCB 136, respectively.

In-cell western

In-cell western was performed pursuant to a protocol provided by LI-COR (Lincoln, NE). Briefly, HEK-293 and the *w_t*RyR1-HEK 293 cells were seeded on 96 well imaging plates (BD life sciences, Franklin Lakes, NJ). The cells were fixed with 4% paraformaldehyde for 25 min and permeabilized with 0.1% Triton-X 100. The cells were then blocked with Odyssey blocking buffer for 1.5 h and incubated overnight with monoclonal antibody 34 C at 4°C. The cells were washed with 0.1% Tween-20 and incubated for 1 h with the secondary antibody goat anti-mouse 800CW (Li-Cor, NE). Finally, the plates were scanned using the LI-COR Odyssey infrared imaging system using the 700 and 800 nm detection channel.

Results

The atropisomers of PCB 136 (Fig 1A) were individually tested for their activity toward enhancing the binding of [3 H]Ry to microsomal membranes enriched with either RyR1 or RyR2. Figure 1B shows that at 26°C and in the presence of low micromolar Ca^{2+} (1 μM), (-)-PCB 136 enhanced the amount of specific [3 H]Ry binding to high affinity sites on the RyR1 complex from nearly undetectable levels to nearly 0.8 pmol/mg protein. Although a similar dose-dependent increase in [3 H]Ry binding was observed when the incubation was performed at 37°C, the dose response was shifted to the right compared to the those obtained at 26°C (EC_{50} s of 1.67 ± 0.11 vs. 0.95 ± 0.19 μM , respectively). (+)-PCB 136 lacked activity toward RyR1 in [3 H]Ry binding assays at either temperature tested. The atropisomers of PCB 136 exhibited the same stereospecificity toward RyR2 as RyR1 when the measurements were made in a buffer containing 200 nM free Ca^{2+} (Fig 1C). Similarly, the relative potency and efficacy of (-)-PCB 136 toward RyR2 was greater at 26°C than at 37°C (EC_{50} of 1.19 ± 0.16 vs. 1.44 ± 0.24 μM ; maximum [3 H]Ry occupancy of 0.152 vs. 0.11 pmol/mg protein, respectively). The nonspecific component of [3 H]Ry to RyR1 and RyR2 preparations was unaffected by the presence of either PCB 136 enantiomer. These data suggest pronounced enantiomeric specificity of (-)-PCB 136 binding sites within RyR1 and RyR2 that are critically important for enhancing the channel open state.

We measured macroscopic Ca^{2+} fluxes across vesicles enriched with RyR1 to further test the hypothesis of enantiomeric specificity toward functional modification of microsomal Ca^{2+} transport. Figure 2A shows that membrane vesicles actively accumulated Ca^{2+} added to the assay buffer in the presence of MgATP. Ca^{2+} accumulation was due to the activity of SERCA pumps, since it was blocked by thapsigargin (TG). Once vesicles accumulated 2×60 nmol of Ca^{2+} added to the extravesicular solution (i.e., the Ca^{2+} load phase), addition of (-)-PCB 136, but not (+)-PCB 136, triggered a release of the Ca^{2+} accumulated by the vesicles (compare traces labeled *(e)* vs. *(c)* in Fig 2A). The actions of (+)-PCB 136 were indistinguishable from those of DMSO solvent control (compare traces *(c)* vs. *(d)*) and were likely the result of basal RyR1 channel activity present under the assay conditions we used. Ruthenium red (RR) is known to block RyRs (28, 31, 35) and is therefore a valid test to determine if PCB-induced Ca^{2+} release from microsomal vesicles is specifically mediated through RyR activation. Traces *(a)* and *(b)* in Figure 2 show that neither enantiomer of PCB 136 influenced baseline Ca^{2+} levels in the presence of RR. RR depressed the baseline below that observed in the DMSO control, indicating that it fully blocked RyR1-mediated Ca^{2+} efflux (compare traces *(a)* and *(b)* vs. traces *(c)* and *(d)*). Addition of TG to block SERCA at the end of the experiment caused a leak of Ca^{2+} from the vesicles regardless of whether or not the RyR1 channels were blocked by RR. (-)-PCB 136 triggered a net efflux of Ca^{2+} from actively loaded microsomal membranes in a concentration dependent range consistent with its activity in the [3 H]Ry binding experiments (0.2–1.0 μM ; Fig. 2B). (+)-PCB 136 lacked efficacy toward triggering Ca^{2+} releasing at concentrations ≤ 5 μM (Fig 2C). It is possible that (+)-PCB 136 could bind to RyR1 at the same site recognized by (-)-PCB 136, without eliciting activation of the channel (i.e., behave as a pure antagonist). Figure 3 shows that the rate of Ca^{2+} released from vesicles triggered by addition of 1 μM (-)-PCB 136 was unaffected by the presence of a molar excess of (+)-PCB 136 (5 μM).

The most direct method of determining if PCB 136 atropisomers directly alter RyR activity and define the underlying mechanisms is to measure gating activity of RyR1 channels reconstituted in BLM (36). Figure 4 shows single channel traces before and after the addition of (+)-PCB 136 (panel A) or (-)-PCB 136 (panel B). Sequential addition of 200 nM and 500 nM (-)-PCB 136 into the cytoplasmic side of the channel (*cis* chamber) elicited a significant 1.8- and 3.0-fold increase in the channel open probability (P_o) without altering the unitary current level. The channel modified by 500 nM (-)-PCB 136 exhibited a 1.7-fold

longer mean open time constant (τ_o) and a 2.5-fold shorter mean closed time constant (τ_c). By contrast, the same concentrations of (+)-PCB 136 had negligible effects on these channel parameters (Fig 4 panel A). Based on results from the [^3H]Ry binding (Fig. 1) and the Ca^{2+} flux (Fig. 2) analyses, we added a saturating concentration (5 μM) of (-)- or (+)-PCB 136 to the *cis* BLM chamber. (-)-PCB 136 (5 μM) enhanced P_o 5-fold, whereas the same concentration of (+)-PCB 136 had a negligible influence on channel gating kinetics (data not shown).

Naïve HEK 293 cells at high passage number did not express detectable levels of immunoreactive RyR protein using in-cell western analysis (Fig. 5A), consistent with previous reports (37, 38). By contrast, an HEK 293 clonal cell line that stably expressed full-length RyR1 showed prominent immunoreactivity with monoclonal 34C (Fig 5A). Verification of RyR1 expression was confirmed with more traditional western blotting of cell homogenates. RyR1-expressing HEK293 showed a single high molecular immunoreactive band at the same position as RyR1 from rabbit skeletal muscle, whereas no immunoreactive band was detected in preparations from RyR-null (not shown).

To assess if the enantiospecific actions of (-)-PCB 136 extend to sensitization of RyR-dependent signaling in intact cells, we exposed RyR-null HEK 293 cells and HEK 293 cells that stably express wild type RyR1 to 100 nM of either enantiomer for 12 hr. The cells were then loaded with the Ca^{2+} indicator Fluo-4 and their responses to caffeine measured using fluorescence imaging in a buffer containing the respective PCB. Figures 5B (trace 4) and 5C show that RyR-null HEK 293 cells failed to respond to 2.5 mM caffeine, even in the presence of (-)-PCB 136. In contrast, cells expressing wild type RyR1 (w_t RyR1; Fig 5A) responded to caffeine with a Ca^{2+} transient whose amplitude is dependent on the concentration of caffeine (Fig 5B, trace 1 and Fig 5C). Importantly exposure to 100nM (-)-PCB 136 (Fig 5B, trace 2), but not (+)-PCB 136 (trace 3), significantly shifted the sensitivity to caffeine-induced Ca^{2+} release in w_t RyR1 to the right (Fig 5C).

Discussion

In this report we provide the first evidence of enantiospecific interaction between an environmentally relevant PCB and a family of microsomal Ca^{2+} release channels known as ryanodine receptors (RyRs). PCB 136 was selected for study because it has been detected in environmental and human tissue samples (5, 9, 11) and there is recent evidence indicates that its two forms undergo enantioselective enrichment and metabolism *in vivo* (24, 25). RyRs represent a family of Ca^{2+} channels that are a direct target of non-coplanar PCBs (39). RyRs are widely expressed in most vertebrate and invertebrate cells where they contribute to local and global Ca^{2+} signals. RyR1 and RyR2 are the major components of the Ca^{2+} release units necessary for excitation contraction coupling of skeletal (40) and cardiac (41) muscle, respectively. Both RyR1 and RyR2 isoforms are broadly expressed in the mammalian brain targeted to discrete locations within neurons where they are responsible for producing Ca^{2+} microdomains necessary for activity dependent growth of dendritic arbors and spines, neurotransmitter release from presynaptic terminals, and influencing synaptic plasticity (42). In the peripheral immune system, RyR1 is expressed in B cells (43) and dendritic cells (44), whereas RyR2 predominates in T cells (45). RyR1 and RyR2 have been implicated in several heritable disorders that confer susceptibility to chemical triggers (46, 47) and stress (40, 48). We have previously shown that non-coplanar PCBs having 2 chlorines in the *ortho*-position are selective sensitizers of RyR1 and RyR2 isolated from muscle (49, 50) and brain (39). A detailed analysis of structure-activity toward RyR1 revealed that the 2,3,6-Cl PCB configuration is optimal for recognition by the RyR1 complex and/or critical for sensitizing its activation (51). *Para* substitutions diminish activity toward channel activation with *para*-chloro having a higher potency than the corresponding *para*-hydroxy derivative.

The addition of a more bulky *para*-methyl-sulfonyl group eliminates the activity toward RyR1, indicating the importance of bulk at the *para*-positions toward influencing RyR1 activity. The structure-activity relationship of PCBs toward RyRs predicts their activity and toxicity in intact cell assays. For example 2,2',3,5',6-pentachlorobiphenyl (PCB 95), the most potent and efficacious congener toward RyRs yet identified, amplifies glutaminergic signaling in cultured cerebellar granule cells, whereas 2,4,4',5-tetrachlorobiphenyl (PCB 66), which has negligible RyR activity, lacks these activities in cultured neurons (52). Likewise 2,2',4,4'-tetrachlorobiphenyl (PCB 47) induces oxidative stress and apoptosis in cultured hippocampal neurons, effects inhibited by the ryanodine receptor (RyR) antagonist FLA 365 and by the antioxidant alpha-tocopherol (53). By contrast coplanar 3,3',4,4'-tetrachlorobiphenyl (PCB 77) lacks these effects (53).

The current results identify for the first time the enantiospecificity with which a chiral noncoplanar PCB binds to, and sensitize the activity of, RyR channels. In this regard, (+)-PCB 136 does not competitively inhibit the Ca^{2+} -mobilizing actions of (-)-PCB 136 indicating that the (+) atropisomer is not capable of competitive binding to the RyR1 complex. The enantiospecificity of (-)-PCB 136 indicates that the spatial configuration of the chlorine substitutions about the biphenyl is significantly more important than the overall physicochemical properties of the PCB for optimizing interactions with RyRs. The current findings also imply a highly ordered binding interaction between active PCBs and their site(s) of interaction within RyR complexes. Previously we have shown that racemic, non-coplanar PCBs interact with the RyR1 complex in a manner that requires its associated accessory protein immunophilin FKBP12 (FK506 binding protein 12 kDa). Dissociation of the FKBP12-RyR1 complex eliminates responses to non-coplanar PCBs while preserving channel responses to other known RyR ligands such as caffeine and ryanodine (50). These results suggest that active PCBs coordinate their binding with RyR1 at a site within or near the clamp region that contains the FKBP12 binding pocket (54, 55). This interpretation is consistent with the known function of FKBP12, which is to stabilize the closed state of the channel (56). PCBs may therefore influence the FKBP12-RyR1 interaction in a manner that destabilizes the closed channel state in favor of the open state. Single channel analyses presented here (Fig. 4) support this interpretation. This interpretation is also consistent with the activity of (-)-PCB 136 in intact in RyR1-expressing HEK 293 that were shown to constitutively express FKBP12 (57).

The results of the current study also raise the intriguing question, whether the enantiospecificity observed with (-)-PCB 136 extends to all 19 chiral PCBs and possibly their corresponding hydroxylated metabolites. Several of the most potent PCB congeners toward RyR channels exist as racemic mixtures including PCB 84 (2,2',3,3',6-pentachlorobiphenyl), PCB 95, PCB 136, and PCB 149 (2,2',3,4',5',6-hexachlorobiphenyl) (51). However, (-)-PCB 84 was only slightly more potent than (+)-PCB 84 at increasing [^3H]-phorbol ester binding and showed no enantiomeric selectivity toward inhibiting $^{45}\text{Ca}^{2+}$ -uptake in preparations of rat cerebellar microsomes (58). Thus the degree of enantioselectivity may be different among chiral PCBs. This hypothesis is supported by [^3H]Ry binding analysis which indicates that (-)-PCB 84 is only 1.6-fold more potent than (+)-PCB 84 toward sensitizing activation of RyR1 (Feng and Pessah, unpublished data). Neither tri nor tetra *ortho*-substituted PCBs would be expected to racemize under physiological conditions, the rotational barriers are simply too high. Thus the substitution pattern around the biphenyl is also important for determining activity toward RyRs. There is also increasing evidence of species-specific enantiomeric enrichment of PCBs, including several human tissue samples (10, 17-19). While PCBs 95, 132 and 149 were near racemic in muscle, brain and kidney samples, an enantiomeric enrichment of PCBs 95, 132, 136 and 149 was observed in the liver. Enantiomeric enrichment has also been documented for several congeners in human breast milk samples from Europe (3). Although the reason for

such observations currently not understood, enantioselective interactions of chiral pollutant enantiomers with biological receptors and enzymes may contribute to species-specific enrichment.

In conclusion, evidence of enantioselective enrichment of PCB atropisomers in environmental samples (4-10), food (3, 11, 12), as well as biological tissues from animals (13-17) and humans (18, 19) has been widely reported. The discovery of enantiospecific activities on biologic targets may have broad significance for human health, not only for the toxic impacts of chiral PCBs.

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(A) 2,2',3,3',6,6'-hexachlorobiphenyl (PCB 136) atropisomers

Figure 1. (-)-PCB 136, but not (+)-PCB 136, enhances the binding of [3H]ryanodine
 PCB 136 atropisomers (A) possess differential biological activities toward the junctional sarcoplasmic reticulum Ca^{2+} channel complexes RyR1 isolated from rabbit or mouse skeletal muscle (B) and RyR2 isolated from rat cardiac ventricle muscle (C) assessed by [3H]ryanodine binding analysis. PCB 136 atropisomers were tested at a concentration range between 0.1 and 10 μM at 37°C for 3 hr or 26°C for 16hr, respectively. The graphs are representatives of a total n=9 independent assays, each individual assay had triplicate samples for every data point.

Figure 2. (-)-PCB 136, but not (+)-PCB 136, triggers Ca^{2+} efflux from microsomal vesicles by selective activation of RyR1

Each cuvette contained 1500 μ l assay buffer including 50 μ g/ml rabbit junctional sarcoplasmic reticulum proteins without or with 1 μ M ruthenium red (RR) pre-incubated for 2 min at 37°C. After the sequential additions of 70 μ M CaCl_2 and completion of active loading of extravesicular Ca^{2+} was complete (downward deflections), the indicated concentration of PCB 136 or solvent dimethyl sulfoxide (DMSO) was introduced into the assay. Upward deflections are caused by the release of sequestered Ca^{2+} released to the extravesicular medium. Approximately 10 min later, either 1 μ M RR, a RyR channel blocker or 200 nM thapsigargin (TG), a SR/ER Ca^{2+} -ATPase blocker, was added to the transport

buffer as denoted in each trace. All the experiments shown were replicated 2 or 3 times with the same results.

Figure 3. (+)-PCB 136 does not compete with (-)-PCB 136 for RyR1 activation

(-)-PCB 136 elicited rapid Ca^{2+} release from actively loaded rabbit junctional sarcoplasmic reticulum vesicles and these effects were not affected by the presence of molar excess of (+)-PCB 136. Upon the completion of the Ca^{2+} loading phase with two bolus additions of 70 μM Ca^{2+} (downward deflections), DMSO (trace **a**), 5 μM (+)-PCB 136 (trace **b**), 1 μM (-)-PCB 136 (trace **c**) or 5 μM (+)-PCB 136 plus 1 μM (-)-PCB 136 (trace **d**) was added to the respective assays. Addition of 200 nM TG approximately 12 min later dissipated the Ca^{2+} gradients. Two additional independent measurements were performed with the same results.

Figure 4. Enantiospecific sensitization of RyR1 channel activity by (-)-PCB 136

Junctional SR isolated from mouse skeletal sarcoplasmic reticulum was used to reconstitute single RyR1 channels in BLM. After recording basal channel gating activity, (+)- or (-)-PCB 136 was introduced into the cytoplasmic (*cis*) side of the channel. Single channel activity was recorded at a holding potential of -40 mV relative to the luminal (*trans*) side of the channel. The open (current conducting) state of the channel is an upward deflection with "O" indicating the maximal open current amplitude. Open probability (P_o), mean open (τ_o) and closed (τ_c) dwell times are denoted above the representative traces. The displayed RyR1 channels are representative of a total number of 8 independent bilayer measurements tested with (+)- or (-)-PCB 136 concentrations of 0.2 to 5 μM .

Figure 5. (-)-PCB 136 selectively sensitizes caffeine responses in RyR1-expressing HEK 293 cells
(A) In-cell western analysis using monoclonal antibody (mAb) 34C that recognizes RyR1 and RyR3. A clone selected for stable expression of RyR1 binds mAb 34C whereas RyR-null HEK 293 cells do not show detectable binding of the mAb. **(B)** Fluo-4 fluorescence emission traces showing Ca^{2+} transient (arbitrary units) responses of individual cells to focal 10 sec application of caffeine with or without 12 hour pre-treatment to PCB 136. **(C)** Summary data of dose-response relationship for caffeine in control (DMSO) and PCB pretreated HEK 293 cells. Data represents dose-response relationships averaged from 10

individual cells. Pretreatment of RyR1-null cells with DMSO, (-)-PCB 136, or (+)-PCB 136 had no influence on responses to caffeine.